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Glycoproteins, process for their preparation and pharmac utical compositions containing them

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Abstract

The invention relates to glycoproteins, process for their preparation, pharmaceutical compositions containing them for stimulating growth and differentiation of human and animal cells of the immune system and for preventing adhesion of leucocytes, platelets and tumour cells to vascular endothelial cells; and for stimulating the immune system, in particular T lymphocytes, to defend against infection, to treat immunodeficiency, oncoses, including metastatic processes, infectious diseases (viruses, bacteria, parasites, protozoa) and circulatory failure, especially vascular occlusions and septicaemias in humans and animals.

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(54) Title: AMINO SUGARS, GLYCOPROTEINS, METHOD OF PREPARING THEM, MEDICINES CONTAINING THEM AND THEIR USE

(54) Bezeichnung: AMINOZUCKER, GLYCOPROTEINE, VERFAHREN ZU IHRER HERSTELLUNG, SIE ENTHALTENDE ARZNEIMITTEL UND IHRE VERWENDUNG

(57) Abstract

The invention pertains to new amino sugars (neuraminic acid precursor analogues) and new glycoproteins, methods of preparing them, pharmaceutical agents containing them and their use in stimulating growth and differentiation of human and animal cells of the immune system and in preventing the adhesion of leucocytes, thrombocytes and tumor cells to vascular endothelium cells, also in stimulating the immune system, especially T-lymphocytes, warding off infections and treating weaknesses in the immune system, tumorous illnesses including metastatic processes, infectious diseases (viruses, bacteria, parasites, protozoa) and circulatory failures, especially vascular occlusions and septicemia, in humans and animals.

(57) Zusammenfassung

Die Erfindung betrifft neue Aminozucker (Neuraminsäure-Vorstufen-Analoga) und neue Glycoproteine, Verfahren zu ihrer Herstellung, sie enthaltende pharmazeutische Mittel und ihre Verwendung zur Stimulierung des Wachstums und der Differenzierung von menschlichen und tierischen Zellen des Immunsystems und zur Verhinderung der Adhäsion von Leukozyten, Thrombozyten und Tumorzellen an Gefäßendothelzellen; sowie zur Stimulierung des Immunsystems, insbesondere von T-Lymphozyten, zur Infektabwehr, zur Behandlung von Immunschwächen, Tumorerkrankungen einschließlich Metastasierungsprozessen, Infektionserkrankungen (Viren, Bakterien, Parasiten, Protozoen) und Kreislaufversagen, insbesondere Gefäßverschlüssen und Septikämien, bei Mensch und Tier.

VERIFIED TRANSLATION OF PCT 0560/94

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Munich, 15.11.94

Dr. Günter Hartmann
European Patent Attorney

English translation

Amino Sugars, Glycoproteins, Method of Preparing Them, Medicines Containing Them and Their Use

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The discovery concerns new amino-sugars (analogues of neuraminic acid precursors) with the illustrated general formula (II), new glycoproteins with the illustrated general formulae (I and I'), methods for their preparation, pharmaceutical preparations containing these amino-sugars and glycoproteins, and the use of these pharmaceutical preparations in humans and animals to: stimulate growth and differentiation of human and animal cells of the immune system; to prevent adhesion of leukocytes, thrombocytes and tumour cells to blood vessel endothelial cells; to stimulate the immune system, in particular T-lymphocytes; to protect against infection; to treat a weak immune response; to treat tumours and the process of metastasis; to treat infectious diseases (caused by viruses, bacteria, parasites and protozoa); to treat circulatory problems, in particular blood vessel occlusion and septicaemia. The discovery also concerns the use of these new substances to inhibit the binding of ligands to their sialylated cell surface receptors; to inhibit the binding of pathogenic microorganisms (viruses, bacteria, parasites, protozoa) or toxins to the sialylated receptors of a host cell, by in vivo modification of neuraminic acids; for the biosynthetic preparation of ligands or receptors containing modified neuraminic acid residues, and the use of these ligands and receptors as competing molecules in physiological or pathological ligand-receptor interactions; to influence, in vitro, the course of infection by human immunodeficiency viruses (e.g. HIV-1 and HIV-2), as well as the in vivo prevention of infection with human immunodeficiency viruses (e.g. HIV-1 and HIV-2), for the treatment of parasitic diseases, in particular trypanosomiasis, leishmaniasis, trichomoniasis, giardiasis, amoebiasis, malaria, pneumocytosis, schistosomiasis (Bilharzia) and echinococcosis.

Glycoproteins are proteins containing covalently bound carbohydrate. In animals, glycoproteins are essential components of cell membranes, as well as soluble constituents of body fluids and the extracellular matrix. The carbohydrates, consisting of single sugar residues or chains (oligosaccharides), are attached to the protein in different ways. Cell membrane glycoproteins contain sialic acids [derivatives of 2-keto-3-deoxy-D-glycero-D-galactononulopyranosidonic acid (KDN)], which play an important part in biological processes.

The oligosaccharides of glycoproteins are classified according to their mode of linkage to the protein. In a high proportion of mammalian glycoproteins, the oligosaccharides are bound *N*-glycosidically to an asparagine residue of the polypeptide chain (*N*-glycans). This group comprises: a) secretory glycoproteins with different functions, e.g. soluble enzymes, immunoglobulins and hormones, and b) membrane glycoproteins, e.g. membrane enzymes, transport proteins and receptor proteins. A further group (*O*-glycans) consists of glycoproteins in which the oligosaccharides are bound *O*-glycosidically to the polypeptide chain via a galactose, *N*-acetylgalactosamine or xylose residue. These occur primarily in mucins, which line the mucosal epithelia of the respiratory, urogenital and gastrointestinal tracts, as well as covering tumour cell surfaces. Together with *N*-glycans, however, they also occur in immunoglobulins and other glycoproteins. The oligosaccharides of proteoglycans, which characteristically contain an especially high proportion of carbohydrate, are also *O*-glycans. In these glycoconjugates, which occur in the extracellular matrix, the oligosaccharides can be bound to the polypeptide chain via a galactose, *N*-acetylgalactosamine or xylose residue.

Glycoproteins are often classified with the glycolipids as glycoconjugates. The sugar components of glycoproteins, which with few exceptions account for less than 50% of the total glycoprotein, are linked to the peptide moiety by *O*- or *N*-glycosidic bonds, which are sensitive to cleavage by glycosidases. Carbohydrates of glycoproteins include hexoses (galactose, mannose, more rarely glucose), *N*-acetylhexosamines, *N*-acylneuraminic acids, fucose and others. Glycoproteins are identified and determined mainly by affinity chromatography, using plant lectins as ligands (e.g. concanavalin A, wheat germ agglutinins, and others).

Almost all membrane proteins, serum proteins, plasma proteins and blood group substances are glycoproteins, as are many enzymes and proteohormones, all antibodies, the chalones, mucins, lectins, bindins, fibronectin, intrinsic factor and similar proteins.

As membrane or cell surface proteins, some glycoproteins play an important part in the pathogenicity of viruses. In this case, as in other receptor-specific cellular interactions, the carbohydrate components are responsible for the recognition process at the molecular level.

Some bacteria and viruses attack their target cells via specific sugar structures of cell surface glycoproteins, which can therefore be regarded as receptors.

Oligosaccharide structures are particularly important in cell-cell and cell-matrix interactions. Thus, the oligosaccharides of glycoproteins mediate the adhesion of neuronal cells and the binding of lymphocytes to specific endothelial cells. Furthermore, oligosaccharides can serve as the antigenic determinants of glycoproteins. Also, during embryogenesis and organogensis, carbohydrate-carbohydrate interactions are essential to specific cell recognition.

Malignant transformation of cells is accompanied by characteristic changes in the oligosaccharide structure of glycoproteins. The extent to which altered oligosaccharide structures of membrane and intracellular glycoproteins of tumour cells are the cause or result of tumorigenesis and metastasis is not known.

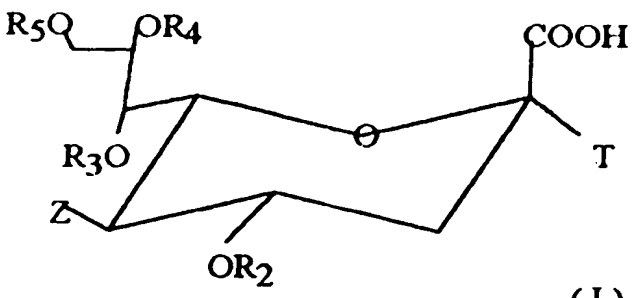
For the treatment of illnesses involving the immune system, substances are required that reinforce the immune response by stimulating the cells of the immune system. The search for active substances that stimulate the immune system is therefore an urgent goal of pharmaceutical research. Active immunostimulants with minimal side effects, however, have so far not been found.

One concern of the present work was therefore to discover substances that can be used to actively and specifically stimulate the immune system.

Surprisingly, our findings showed that these requirements are met by new glycoproteins with the illustrated general formulae (I and I') and new amino-sugars (analogues of neuraminic acid precursors) with the general formula (II), which stimulate proliferation of animal and human cells of the immune system.

Subject of the discovery are new glycoproteins, with the general formulae (I) and (I'), and new amino-sugars (analogues of neuraminic acid precursors) with the general formula (II):

(Neo)glycoproteins with the general formula (I)



(I)

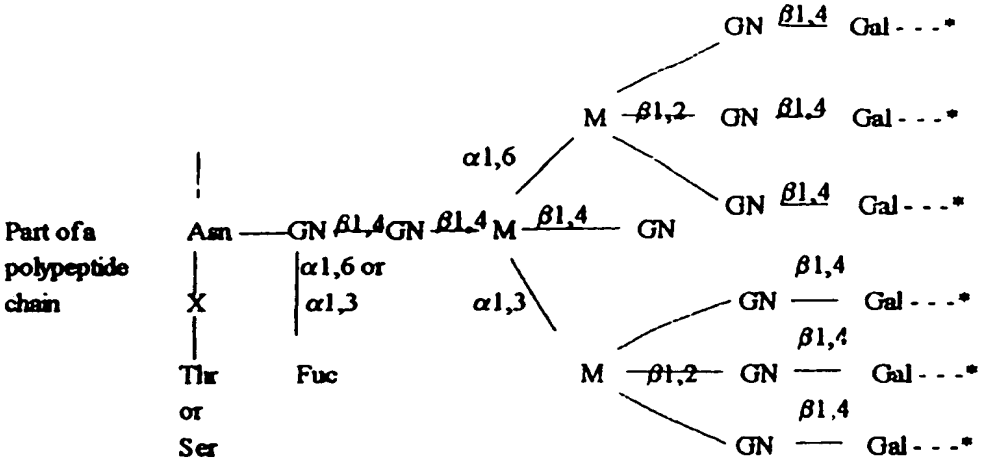
where:

Z represents $-R_1$, $-NH_2$, $-NHR_1$, $-NR_1R_{1'}$, $-N^+R_1R_{1'}R_{1''}$, $-OR_1$, $-SR_1$ or $-CR_1R_{1'}R_{1''}$;

R_1, $R_{1'}$, $R_{1''}$, R_2, R_3, R_4 and R_5 can be the same or different; each of these groups can be a hydrogen atom; a linear or branched alkyl residue with 1 to 20 carbon atoms (C_nH_{2n+2}, where n = 1 to 20), usually 1 to 7 carbon atoms; a linear or branched alkenyl residue with 3 to 20 carbon atoms (C_nH_{2n}, where n = 3 to 20; double bond on C2 to 19), usually 3 to 10 carbon atoms; a linear or branched alkynyl residue with 3 to 20 carbon atoms (C_nH_{2n-2}, where n = 3 to 20; triple bond on C2 to C19), usually 3 to 10 carbon atoms; an alkenyl or alkynyl residue with 2 or more double or triple bonds, with 4 to 10, usually 7 to 12 carbon atoms; an aryl residue with 6 to 20 carbon atoms, usually a phenyl residue; a linear or branched, saturated or mono- or multi-unsaturated acyl residue ($-CO-R_1$), with a total of 1 to 20, usually 1 to 7 carbon atoms, including its mono- or multi-hydroxylated analogues; an aroyl residue with 6 to 20, usually 6 to 10 carbon atoms; a carbonylamide residue of the formula $-CONH_2$, $-CONHR_1$, $-CONHR_1R_{1'}$, or $-CONR_1R_{1'}R_{1''}$; a linear or branched, saturated or mono- or multi-unsaturated thioacyl residue ($-CS-R_1$), with a total of 1 to 20, usually 1 to 7 carbon atoms; or a thiocarbamide residue of the formula $-CS-NH_2$, $-CS-NHR_1$, $-CS-NR_1R_{1'}$, or $-CS-NR_1R_{1'}R_{1''}$; whereby each of the aforementioned residues, with the exception of H, can be singly or multiply substituted by halogens (fluorine, chlorine, bromine or iodine) and by hydroxy-, epoxy-, amino-, mercapto-, phenyl-, phenol or benzyl groups;

T is a mono-, di- or oligosaccharide residue (containing 5 to 230 carbon atoms) with up to 40 glycosidically linked, unbranched or branched sugar residues (with furanose and/or pyranose rings), linked *N*- or *O*-glycosidically to the polypeptide.

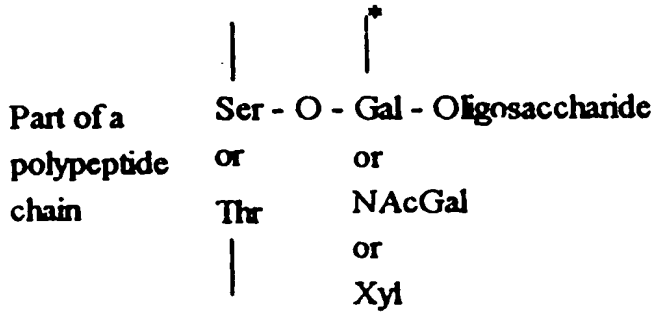
With respect to the present discovery, particularly important glycoproteins are those with the illustrated formula (I), in which T is either:
an N-glycan with the formula (Ia):



(Ia)

where
Gal = galactose,
GN = N-acetyl-D-glucosamine,
M = D-mannose,
Fuc = fucose,
Asn = asparagine,
X = any amino acid except proline,
Thr = threonine,
Ser = serine
* = attachment site of T (1 to 6 residues),
and where both peripheral M residues can be substituted by 1 to 3 tricaccharides;

or in which T is a saccharide residue with O-glycan structure with the general formula (Ib):



(Ia)

where

Gal = galactose,

Thr = threonine,

Ser = serine,

Xyl = xylose,

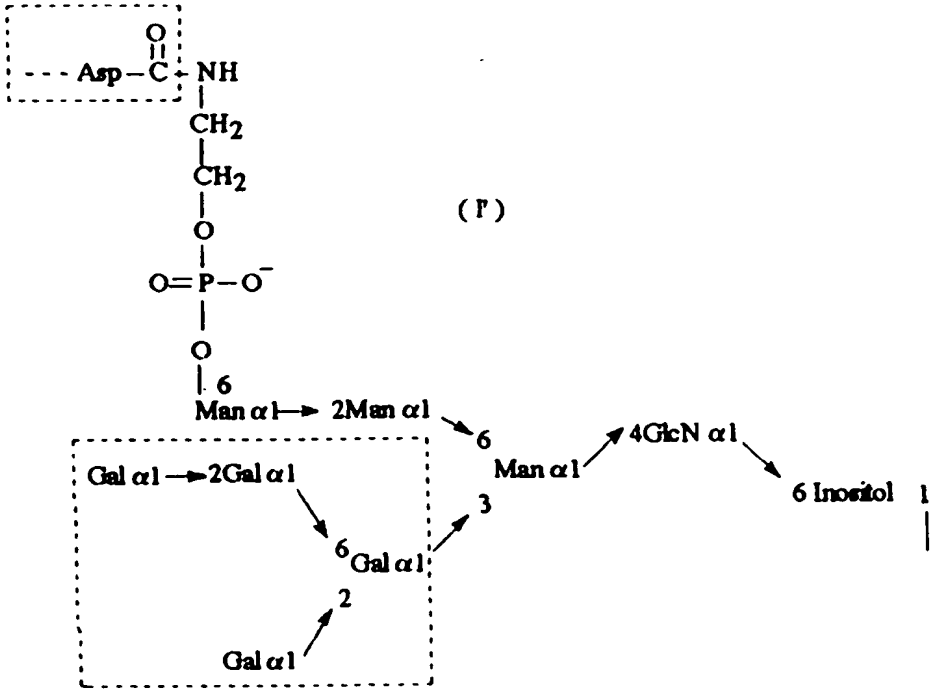
NAcGal = N-acetylgalactosamine,

* = attachment site of T,

and where in the above formulae (Ia) and (Ib), galactose (Gal) can be replaced by 2-deoxy-galactose or 2-deoxy-halogenide (F, Cl, Br, I)-galactose;

or in which T is represented by a

(Neo)glycoprotein with the general formula (I'), which serves as a glycosylphosphatidyl inositol (GPI) anchor:



(I')

where

Gal = galactose,

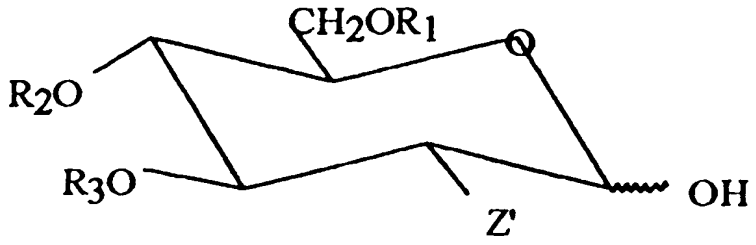
Man = mannose,

Asp = asparagine,

GlcN = glucosamine,

and where GlcN can be replaced by an analogue of a neuraminic acid precursor (formula II) of the type described in this patent.

Glycoproteins of particular relevance to this discovery are those with the formula (Ia), and in which T is linked *N*-glycosidically or *O*-glycosidically to the polypeptide, and in which GN is a residue with the general formula (II):



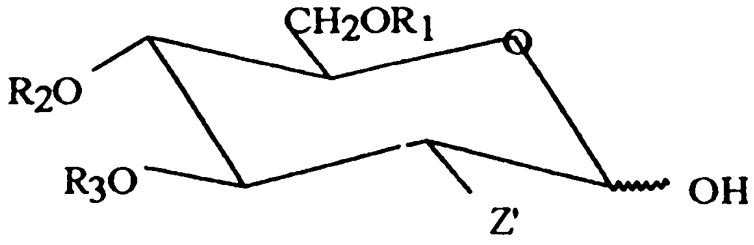
(II)

where R_1, R_2 and R_3 are the same as already listed above, and Z' is the same as Z. Furthermore, Z' can ocupy the equatorial position or the axial position, and when it occupies the equatorial position, the axial position can be occupied by $-OR_2$.

Glycoproteins of very great relevance to this discovery are those with the formula (Ia), in which GN is a residue with the general formula (II), and in which Z or Z' is NHR_1, where R_1 is represented by a propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl or crotonoyl group, or by a mono- or multi-hydroxylated analogue of one of these groups.

Glycoproteins of very great relevance to this discovery are also those with the formula (Ia), in which GN is a residue with the general formula (II), and in which the groups R_2 to R_5 are H or CN_3.

Amino-sugars (analogues of neuraminic acid precursors)



(II)

in which Z', R_1, R_2 and R_3 are the same as already listed above.

The novel amino-sugars (general formula II) of relevance to this discovery and the (neo)glycoproteins (general formulae I and I') represent new classes of substances which are potent agents for the treatment of illnesses involving cells of the specific and unspecific immune defence systems, tumour cells, leukocytes, thrombocytes and blood vessel endothelial cells. These compounds directly interact with, and modulate, membrane receptors which are involved in the regulation of growth and differentiation, and in the adhesion of cells of the immune system, tumours cells and blood vessel cells. Immunostimulation is necessary for the cure of infectious illnesses and tumours. In addition, these compounds prevent adhesion of leukocytes or tumour cells to blood vessel endothelial cells in septic shock or in metastasis, respectively. Administration of these substances, in particular those of formula (II), causes no detectable side effects.

The novel amino-sugars relevant to this discovery (II) and the novel immunostimulants, glycoproteins type (I) and (I'), can be used to stimulate cells of the immune system. By administration of these compounds, it is possible to reinforce the immune system of organisms that have a weak immune response. These compounds are characterised by high cell specificity and the absence of side effects.

The residues named in formulae (I), (I') and (II) are described in greater detail as follows.

Examples of the above-mentioned alkyl residues with 1 to 20, usually 1 to 7 carbon atoms, are propyl, butyl, pentyl, hexyl, isopropyl and pivalinyl residues.

Examples of appropriate alkenyl residues are propenyl, but-2-enyl, but-3-enyl, pent-2-enyl and pent-3-enyl residues.

Examples of appropriate alkenyl residues with more than one double bond are pent-2,4-dienyl and hex-2,4-dienyl residues.

Examples of appropriate substituent groups are chloro, ethyl, dichloroethyl, trichloroethyl, *p*-chlorophenyl, hydroxymethyl, 3-hydroxypropyl and *p*-hydroxyphenyl groups.

Sialic acid is a component of type (I) glycoproteins and type (II) amino-sugars.

Examples of long-chain acyl residues are caproyl, octoyl, lauroyl, myristoyl, palmitoyl, stearoyl, oleyl, linoleyl, linolenoyl and arachidonyl residues, as well as their mono- or muli-hydroxyated analogues.

Examples of appropriate monosaccharide, disaccharide and oligosaccharide residues are D-glucosyl, D-galactosyl, D-mannosyl, xylosyl, inosyl, ribosyl, arabinosyl, fructosyl, sorbosyl, lactosyl, saccharosyl, trehalosyl, maltosyl, cellobiosyl and higher saccharide residues like raffinosyl, fucosyl, chitobiosyl, chitobiosemannosyl, rutinosyl and rhamnosyl residues. Both 1α and 1β glycosidic linkages are possible. One or more sugar rings can be present as sugar amines, such as glucosamine, mannosamine and galactosamine.

Also a subject of this patent are methods for the in vivo preparation of compounds with formulae (I), (I') and (II), by the parenteral or enteral administration, to humans or animals, of a 2-deoxy-2-amino-mannose, -glucose or -galactose, in which the amino group is substituted by R_1, usually by *N*-propanoyl-, *N*-butanoyl-, *N*-pentanoyl-, *N*-hexanoyl-, *N*-heptanoyl- or *N*-crotonoyl-D-mannosamine.

This patent also includes the therapeutic application of glycoproteins with the general formulae (I) and (I'), which are synthesized in vivo and prepared (separated) by known methods.

Also a subject of this patent are pharmaceutical agents:

for stimulation of the immune system, in particular stimulation of T-lymphocytes, protection against infection, treatment of a weak immune response, treatment of tumours, including the process of metastasis, treatment of infectious diseases (caused by viruses, bacteria, parasites, protozoa), treatment of circulatory problems, especially blood vessel occlusion and septicaemia, all in humans and animals;

for increasing the cytotoxic activity of natural killer cells (NK-cells), in order to induce an antitumour immune reaction in humans and animals;

to increase the phagocytic activity of granulocytes and monocytes, in order to induce an antitumour reaction in humans and animals;

to modulate neuraminic acid-dependent biological processes in vivo;

to inhibit ligand binding to sialylated cell surface receptors (endothelial cells, thrombocytes, leukocytes);

to inhibit the sialylated receptor-mediated binding of pathogenic microorganisms (viruses, bacteria, parasites, protozoa) or their toxins to host cells, by in vivo modulation of neuraminic acids;

in which at least one active component is a compound with the general formula (I), (I') or (II), or is a product of the described preparation procedures, if necessary in combination with other active substances, as well as the usual pharmaceutical vehicles and/or auxiliary substances.

The pharmaceutical preparations relevant to this patent contain compound(s) of formulae (I) and/or (I') and/or (II) and/or the products of preparative methods in a quantity of 0.01 to 50% by weight, usually from 0.1 to 20% by weight, and in particular from 2 to 10% by weight of the pharmaceutical preparation.

Also a subject of this patent is the use of compounds of the general formulae (I) and/or (I') and of the general formula (II) and/or products of the described preparative procedues:

to stimulate the growth and differentiation of human and animal cells of the immune system and to prevent adhesion of leukocytes, thrombocytes and tumour cells to the endothelial cells of blood vessels;

to stimulate the immune system, in particular T-lymphocytes, to protect against infection, for the treatment of a weak immune response, for the treatment of tumours and the process of metastasis, for the treatment of infectious diseases (viral, bacterial, parasitic and protozoal diseases), for the treatment of circulatory problems, in particular blood vessel occlusion and septicaemia, in humans and animals;

to inhibit the binding of ligands to their sialylated cell surface receptors;

to inhibit the sialylated receptor-mediated binding of pathogenic microorganisms (viruses, bacteria, parasites, protozoa) or their toxins, by the in vivo modulation of neuraminic acids;

for the biosynthetic preparation of ligands or receptors containing modified neuraminic acid, and the use of these ligands or receptors to bring about competitive physiological or pathological ligand-receptor interactions;

to modulate in vitro the course of infection by human immune deficiency viruses (e.g. HIV-1 and HIV-2);

to prevent infection in vivo by human immune deficiency viruses (e.g. HIV-1 and HIV-2); and

for the treatment of parasitic diseases, in particular trypanosomiasis, leischmaniasis, trichomoniasis, giardiasis, amoebiasis, malaria, pneumocytosis, schistosomiasis (Bilharzia) and echinococcosis.

An important area of application of the present discovery is the use of analogues of neuraminic acid precursors for the in vivo modulation of receptor-ligand interactions that involve neuraminic acids.

As is generally known, an important property of protein-bound carbohydrates is that they stabilize the entire molecule (i.e. the glycoprotein) and protection it from degradation [cf. Ashwell and Harford (1982) *Annual Review of Biochemistry 51*, 531-554; and Tauber and Reutter (1989) *Futura 2*, 11-18 (in particular scheme on p.13)]. It is also known that changes in the oligosaccharide structure lead to changes in the biological stability of the glycoprotein, as measured by its biological half-life. This is the case for the majority of glycoproteins that have been studied.

Glycoproteins (naturally synthesized glycoproteins, and glycoproteins prepared by gene technology, biochemical methods or chemical methods) can therefore be specifically altered by modifying their oligosaccharide structures. Such modifications can be used to produce glycoproteins with therapeutic effects. This is of particular importance for the following classes of compounds:

a) <u>Bile acids</u>

The amino-sugars of this patent (analogues of neuraminic acid precursors) with the general formula (II) can be coupled with bile acids in vivo (in particular with the sterane ring of the bile acids) to form bile acid glycosides. Coupling with the *N*-acylhexosamines, *N*-propanoyl-, *N*-butanoyl-, *N*-pentanoyl-, *N*-hexanoyl-, *N*-heptanoyl-

and *N*-crotonoylhexosamines, in particular the corresponding glucosamines and galactosamines, is clinically beneficial.

Bile acids are excreted in large part as glycosides, e.g. as *N*-acetylglucosaminide. The formation of bile acid hexosaminides is increased by the administration of *N*-acyl-D-glucosamines. Since an increase in the blood concentration of bile acids (synthesized in the liver) leads to agonizing itching in many patients with liver diseases, the administration of *N*-acylhexosamines (as listed above) increases the renal elimination of bile acids and thereby decreases the itching.

b) Mucins

These mucopolysaccharides are glycosaminoglycans, and they have an important protective function in the bronchial system, the gastrointestinal tract, and the urogenital system; their *N*-acetyl-hexosamine components, as well as their *N*-acetyl- or *N*-glycolyl-neuraminic acid components can be partly or completely replaced by the *N*-acylhexosamines described in this patent or by their metabolic products (*N*-acylneuraminic acids) by in vivo coupling to mucins.

The *N*-acetylhexosamine components of the naturally occurring substances can be partly or completely replaced by the *N*-acylhexosamines listed in this patent, usually by the *N*-acyl-galactosamines and -glucosamines.

The *N*-acetyl- or *N*-glycolyl-neuraminic acid components of mucins can be partly or completely replaced by the *N*-acyl-derivatives listed in this patent, by the in vivo administration of the *N*-acylhexosamines listed in this patent, in particular the *N*-acyl-mannosamines or -glucosamines.

The physical-chemical properties of mucins can be altered by the administration of the amino-sugars (analogues of neuraminic acid precursors) listed in this patent, which have the general formula (II). For example, in bronchitis, an improvement is achieved by increasing the solubility of the mucins and therefore the ease of removal of mucus by coughing. Similarly, the mucus lining of the gastrointestinal tract is stabilized, thus increasing the protection of the intestinal mucosa against harmful dietary substances and the hydrochloric acid that is produced endogenously in the stomach.

c) Glycosamines such as hyaluranic acid, produced either by gene technology, or by biochemical or chemical methods; sulphated glycosaminoglycans such as heparin, chondrotin sulphate, dermatan sulphate or keratan suphate; and proteoglycans such as collagens.

These substances can be used, for example, for the treatment of skin complaints (dry skin, wounds, neurodermatitis, ulcers). The *N*-acetylglucosamine or *N*-acetylgalactosamine components of these naturally structures can be replaced by *N*-acylhexosamines listed in this patent, in particular by *N*-propanoyl-, *N*-butanoyl-, *N*-pentanoyl-, *N*-hexanoyl-, *N*-heptanoyl- and *N*-crotonoyl-galactosamines and -glucosamines.

If these substances (prepared by gene technology, or by biochemical or chemical methods) also contain *N*-acetyl- or *N*-glycolylneuraminic acid, these groupings can also be partly or completely replaced biologically by the in vivo administration of the *N*-acylhexosamines listed in this patent, in particular by *N*-acylmannosamines and *N*-acylglucosamines. Chemically, this replacement is performed by deacylation, followed by reacylation, for example with propionic acid anhydride. Biochemically, it is performed by in vivo modulation (as described above) by administering the appropriate *N*-acyl-D-mannosamine precursor (for example, *N*-propanoylmannosamine is given, in order to generate *N*-propanoylneuraminic acid).

d) Glycoproteins such as hormones, hyaluronic acid and its sulphated derivatives (as described above), prepared by gene technology, or by biochemical or chemical procedures.

Hormones, like erythropoetin (important for blood formation; deficient in dialysis patients), growth hormone (hCG = human growth hormone), and tissue hormones (also called mediators) like interleukins (at least 10 interleukins have been described) are synthesized by cells of the lymphatic system and by macrophages. Interleukin 6 is synthesized by the liver. This group also includes antibodies and interferons (α-, β-, γ-interferons and their subtypes) for the treatment of viral diseases and of tumours.

Also in these substances, the constituent *N*-acetylglucosamine or *N*-acetylgalactosamine components can be replaced by the *N*-acylhexosamines listed in this patent, in particular by *N*-acylglucosamines and *N*-acylgalactosamines, by administration of the amino-sugars (II) listed in this patent.

Their *N*-acetylhexosamine components can be completely or partly replaced by *N*-acylhexosamines, in particular *N*-acylglucosamines and *N*-acylgalactosamines. The constituent *N*-acetylneuraminic acid components can be replaced, however, by the corresponding *N*-acylneuraminic acids which are formed in vivo after administration of the *N*-acylhexosamines listed in this patent, in particular *N*-acylmannosamines and *N*-acylglucosamines.

The acyl residues referred to in the present work are mainly propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl and crotonoyl residues, as well as their mono- or multi-hydroxylated analogues.

The hexosamines referred to in the present work are in particular glucosamines, galactosamines and mannosamines.

Many biological processes (for example, the action of hormones, interleukins and pharmaceutical agents, and cell-cell recognition) are based on the interaction of ligands with receptors on cell surfaces. Pathogenic microorganisms (viruses, bacteria, protozoa) or their toxins frequently exert their pathogenic action by exploiting cell surface structures as receptors for interaction with host cells. Cell-cell recognition is intimately involved in physiological and pathological (e.g. autoimmune diseases) functions of the immune system, in tissue differentiation and in the immunological control of malignant tumour cells.
Carbohydrate structures play an important part in a large number of these ligand-receptor interactions. The terminal neuraminic acid residues of these carbohydrate structures play a special role in ligand-receptor interaction; for example the influenza virus uses a sialylated cell surface receptor. Sialylated differentiation antigens are necessary for the the activation of T-lymphocytes. In addition, different forms of cell surface sialylation have been reported as determinants for the progression and metastasis of malignant tumours.

It is possible to influence neuraminic acid-dependent biological processes by using non-toxic analogues of neuraminic acid precursors, which are taken up by the cell, then incorporated metabolically into carbohydrate structures in place of endogenous neuraminic acid. The resulting modified neuraminic acid residues may weaken, reinforce, or have no effect on ligand-receptor interactions.
This weakening (A) or intensification (B) of receptor-ligand interaction is illustrated by the following two examples:

A. Reduction in the susceptibility of human B-lymphoma cells (BJA-B) to infection by the lymphotropic papovavirus (LPV) by pretreatment of the cells with *N*-propanoyl-D-mannosamine or *N*-butanoyl-D-mannosamine.
The BJA-B receptor used by LPV contains neuraminic acid which is essential to the interaction; thus, treatment of the cells with neuraminidase from *Vibrio cholerae* decreases virus binding and susceptibility to infection more than five-fold (own unpublished results).
B. Increase in the susceptibility of simian kidney epithelial cells (Vero) to infection by human polyomavirus BK (BKV) by pretreatment of the cells with *N*-propanoyl-D-mannosamine.

A cell surface-bound neuraminic acid is essential for the infection of Vero cells by BKV, since preincubation of Vero cells with neuraminidase decreases their susceptibility to infection [Sinibaldi et al. (1990) *Arch Virol.* ***113***, 291-296].

MATERIALS AND METHODS

1. Abbreviations used

A	Absorption
BKV	BK virus
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DTT	1,4-Dithiothreitol
EBV	Epstein-Barr virus
ELISA	Enzyme-coupled immunosorbent test
FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
LPV	Lymphotropic papovavirus
LRSC	Lissamine-rhodamine isothiocyanate
mAb	Monoclonal antibody
OD	Optical density
PBS	Phosphate-buffered saline
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SV40	Simian virus 40
TMB	Tetramethylbenzidine
TRITC	Tetra-rhodamine isothiocyanate
VP	Viral structural protein
FACS	Fluorescein-activated cell sorter
Scan	Scanner

2. Cell culture media

The cell culture media used in this work were RPMI 1640 and DMEM, obtained as dry preparations from Biochrom, Berlin. For use, they were reconstituted according to the manufacturer's instructions in pyrogen-free H_2O, and sterilized by filtration. The following additions were made: 10% FCS (incubated for 30 min at 56°C to inactivate the complement system), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

3. Buffers

PBS (pH 7.2): 125 mM NaCl (7.2 g); 18.4 mM Na_2HPO_4 (3.14 g); 10.9 mM KH_2PO_4 (1.42 g); made up to 1 litre with H_2O.

"Extraction buffer" for the preparation of LPV and BKV inoculation virus from infected cultures:

50 mM HEPES pH 7.4; 1 mM $MgCl_2$; 0.5 mM $CaCl_2$; 1 mM DTT; 1 mM PMSF; 2.5 µg/ml amphotericin B; 200 µg/ml gentamycin; 200 U/ml penicillin; 200 µg/ml streptomycin.

DTT, PMSF, amphotericin B, gentamycin, penicillin and streptomycin are added fresh to the autoclaved buffer before use.

4. Cell lines

The human B-lymphoma cell line BJA-B [Menezes et al. (1975) *Biomedicine 22,* 276-284] was used as the LPV-susceptible cell line. This cell line is obtainable from the European Collection of Animal cultures (ECACC), Porton Down, Salisbury (UK), where it is deposited according to the conditions of the Budapest convention under number 86081105. The simian kidney epithelium cell line, Vero, was used as the BKV-susceptible cell line. This cell line is obtainable from the American Type Culture Collection (ATCC), Rockville, Maryland (USA) (catalogue number ATCC CRL 1587).

5. Cell culture

All cell culture work was conducted at a safety work-bench (L II). Cells were maintained in incubation cabinets at 37°C with 5% CO_2 and 95% air humidity. BJA-B cells were cultured in glass Erlenmeyer flasks with aluminium foil caps. Between cell densities of 2×10^5 and 1×10^6 cells per ml, the cells were in the logarithmic phase of growth. Cells that had reached the stationary growth phase were diluted 1:3 - 1:10 with fresh growth medium, and growth was allowed to continue [Forbes et al. (1988) *Mol. Cell. Probes 2,* 245-253]. The adherent monolayer cell line, Vero, was maintained in plastic cell culture flasks. Upon the attainment of confluent growth, the medium was removed, and the cell lawn was washed with a few ml of trypsin solution (0.1% trypsin; 0.1% EDTA in H_2O). After brief exposure to trypsin at 37°C, the Vero cells detached from the substratum. They were then diluted 1:3 - 1:20 with fresh medium [Mühlbach et al. (1992) *Virology 186,* 65-73].

For the determination of cell concentration, 100 µl of suspended cells were mixed with 100 µl of trypan blue solution (0.25% trypan blue in PBS), and a few drops were pipetted between the cover glass and chamber of a Neubauer counting chamber. Dead cells were stained blue and could be differentiated from the brightly luminous living cells under the light microscope. The sum of cell counts from two of the four quadrants, multiplied by 10^4, gave the number of cells per ml of the original culture.

6. Antisera and monoclonal antibodies

The following were used: a polyclonal hamster-α-LPV-T-antigen serum; a polyclonal rabbit-α-LPV-VP-serum; a monoclonal antibody (mAb) 456-1 from mouse ascites, raised against LPV-VP1 (from M. Pawlita, Heidelberg); a mouse-mAb against SV40-T-antigen (mAb SV1-3H9) (from F. Mehnert, Bochum); and a rabbit antiserum against BKV particles (from G. Noss, Rehlingen-Siersburg).

7. Preparation of inoculation virus

7.1 Preparation of LPV inoculation virus

Lymphotropic papovavirus (LPV) is obtainable from the American Type Culture Collection (catalogue number ATCC VR-961). Between 200 and 300 µl of an LPV inoculation virus solution were added to 2×10^7 BJAB cells in culture. Every 3 days the culture volume was increased five-fold by the addition of fresh medium. From the 6th day the percentage of virus-infected cells was determined by indirect immunofluorescence. The culture was harvested (centrifugation for 15 min at 600 g) when it contained between 30 and 60% virus-infected cells. The sedimented cells were washed in PBS, then frozen at -20°C for cell lysis. To extract the virus, the cell sediment was resuspended in cold "extraction buffer" (1/20 of the cell culture volume), and kept on ice for 60 min with frequent whirling (vortexing). Cell debris was then removed by centrifugation for 10 min at 2,500 g and 4°C. Using extraction buffer, the sediment was re-extracted for 5-10 min with 1/60 of the original cell culture volume, followed by centrifugation; this re-extraction procedure was performed three times, and the four supernatants were combined. The infectivity of the LPV suspension was determined by titration on BJA-B cells, and the inoculation virus was stored frozen at -20°C until use.

7.2 Preparation of BKV inoculation virus

Human polyomavirus BK (BKV) is obtainable from the American Type Culture Collection (catalogue number ATCC VR-837). BKV inoculation virus was obtained from infected Vero cells that had been cultured for about 3 weeks. Cells were detached with the aid of trypsin, sedimented by centrifugation for 15 min at 600 g, washed in PBS, and frozen at -20°C for cell lysis. The cell sediment was submitted to two cycles of freezing and thawing, then resuspended in cold "extraction buffer" (1/20 of the cell culture volume) for virus extraction. This suspension was kept on ice for 60 min with frequent whirling (vortexing), then cell debris was removed by centrifugation for 10 min at 2,500 g and 4°C. The infectivity of the supernatant was determined by titration on Vero cells, and the inoculation virus was stored frozen at -20°C until use.

8. Treatment of cells with neuraminidase, and subsequent virus infection

BJA-B cells (1.5×10^6) were washed twice in PBS, kept for 1 h in PBS at 37°C, then incubated with 20 mU neuraminidase (from *Vibrio cholerae)* in 100 ml PBS (i.e. 0.2 U/ml neuraminidase) for 60 min at 37°C with shaking. After washing once in PBS (4°C, 400 g, 10 min), the cells were incubated again with neuraminidase (0.2 U/ml) under the same conditions. After a further 60 min, the cells were resuspended in cold medium, then sedimented by centrifugation for 10 min at 2,600 rpm and 4°C. The cell sediment was mixed with 400 ml of LPV inoculation virus suspension, and incubated at 4°C for 3 h with frequent whirling. Unbound virus was removed by washing once with medium, and the cells were cultured for 48 h in 2 ml of fresh medium.

Vero cells that had grown to about 50% confluency on cover glasses were washed with PBS, then treated with neuraminidase (from *Vibrio cholerae),* as described above, for a total of 2 h. The enzyme solution was removed by washing with culture medium, after which the cells were incubated with BKV inoculation virus for 3 h at 4°C. Unbound BKV was then removed from the Vero cells by washing three times with culture medium. After culturing for 48 h at 37°C, the percentage of virus-infected cells was determined by indirect immunofluorescence.

9. Treatment of cells with *N*-acyl-D-mannosamines. and subsequent infection with LPV or BKV

BJA-B cells were suspended in fresh medium at a concentration of 1×10^6 cells/ml for treatment periods of 3 h, and at a concnetration of 2×10^5 cells/ ml for 48 h incubation. The concentration of the neuraminic acid precursor analogues in the PBS was varied between 0.01 and 10 mM. Monolayer cells were sown at an appropriate density on cover glasses, so that about 4 days were required for the attainment of confluent cell growth.

After treatment with the neuraminic acid precursor analogues, the cells were washed in PBS, then incubated with inoculation virus for 3 h at 4°C. These LPV-treated BJA-B cells were washed once with medium, then sedimented by centrifugation. BKV-treated Vero cells were washed three times with medium. After a further 48 h in culture, the percentage of virus-infected cells was determined by indirect immunofluorescence or by LPV permissiveness, respectively.

10. Measurement of virus binding and virus infection

(i) Determination of the degree of virus infection

The degree of infection of cultures by LPV was determined in two ways: by measuring the percentage of virus antigen-producing cells by indirect immunofluorescence (Forbes

et al., as quoted above), and by quantifying the main structural protein of the virus, LPV-VP1 [with an ELISA (see 10.2.3.)] relative to total protein in an extract of infected cells. In the following, this quotient (expressed as ng LPV-VP1 per mg total protein) is called the LPV permissiveness.
The degree of infection by BKV was likewise determined by indirect immunofluorescence, using a rabbit antiserum against BKV particles.
(ii) LPV binding test
The binding of LPV particles to BJA-B cells was measured as described under 10.3.

10.1. Indirect immunofluorescence

To determine the percentage of virus-infected cells, 5×10^5 cells were harvested by centrifugation 48 h after infection, and resuspended in 50 µl PBS (1×10^4 cells/µl). Of this suspension, 10 µl were placed on one field of an adhesion microscope slide coated with poly-L-lysine (BioRad adhesion slides, BioRad, Munich). The slide was placed in a humid chamber, and the cells sedimented onto the poly-L-lysine substratum within 30 min. Non-attached cells were washed away with PBS, and the slide finally fixed for 5 min in an acetone/methanol mixture (1:1) at -20°C.
Vero cells were sown on cover slips (diameter 10 mm), allowed to grow, washed once with PBS, then fixed as described above.
Using an humidified chamber, fixed cell preparations were incubated at 37°C for 45-60 min with 10-30 µl/field of monoclonal or polyclonal first antibodies. Finally, the slide was carefully washed for 5 min in a dish of PBS, with magnetic stirring. The second reagents were commercially available fluorochrome-coupled (FITC, TRITC, LRSC) anti-immunoglobulins (diluted 1:50 - 1:100 in PBS). DAPI was added to the second reagent to stain nuclei. After a further 45-60 min incubation in the dark, followed by washing, the preparations were embedded in an elvanol solution under cover slips (24 x 60 mm) with the exclusion of air bubbles, and stored at 4°C protected from light.
[Preparation of elvanol solution: 20 g elvanol (polyvinylalcohol), 160 ml PBS, 80 ml glycerol; the solution is kept at 80°C until the elvanol is completely dissolved, then autoclaved in separate portions].
Fluorescence microscopy was performed with a Vanox-T microscope from Olympus, T kio, Japan.

10.2.1. Extraction of infected cells for the determination of LPV permissiveness

Cells ($3\text{-}4 \times 10^6$) were harvested 48 h after infection, washed in PBS, and the cell sediment frozen for at least one day at -20°C for cell lysis. Virus extraction was carried out as described under 7.1: the cell sediment was resuspended in 400 µl of cold "extraction buffer", and kept on ice for 60-90 min with frequent whirling. Cell debris

was then removed by centrifugation for 10 min at 4,500 g and 4°C. The protein concentration (see 10.2.2.) and the concentration of LPV-VP1 (see 10.2.3.) were determined in the virus-containing supernatant, and LPV permissiveness was calculated from the results.

10.2.2. Determination of the protein concentration of the cell extract

The protein concentration of the extract of infected cells was determined with the "BioRad-microassay" according to the instructions of the manufacturer. Sufficient protein solution was added to generate a blue colour corresponding to that generated by between 10 and 20 µg of the protein standard provided by the manufacturer. The exact protein concentration of the sample was interpolated from the standard calibration.

10.2.3. LPV-VP1 ELISA

Affinity-purified mAb 456-1 against LPV-VP1 was diluted 1: 10^4 in coupling buffer [0.05 M Na_2CO_3 (pH 9.6)] and 100 µl placed in each well of an ELISA plate (96-well plate, NUNC, Wiesbaden). After storage at 4°C overnight, the plates were washed (5 x 300 µl per well) with washing buffer (PBS containing 0.05% Tween 20) in an Ultrawash II apparatus (Dynatech, Denkendorf) to remove unbound antibodies. Remaining free binding sites were blocked by placing 200 µl of 0.2% gelatin solution (in PBS containing 0.1% sodium azide) in each well and incubating at RT for 2 h. The plates could then be stored for several weeks at 4°C until used.

(1) LPV suspensions from extracted cells were diluted 1:2 to 1: 2000 in PBS, and 100 µl of this diluted suspension added to each well. For construction of the calibration curve, a purified particle preparation (5 mg LPV-VP1/µl) was diluted to give a series of solutions containing 12.5 pg to 1.6 ng LPV-VP1 per 100 µl per well. Incubation was performed for 60 min at 37°C.

(2) The plate was washed thoroughly with washing buffer (9 x 300 µl per well).

(3) The polyclonal rabbit serum against LPV-VP was diluted 1: 25,000 in washing buffer, and 100 µl of this solution were added to each well as second antibody.

(4) The plate was washed again with washing buffer (9 x 300 µl per well).

(5) Peroxidase-coupled goat antirabbit IgG was diluted in washing buffer (1:5000) and 100 µl of this solution was added to each well of the ELISA plate. The plate was then incubated for 60 min at 37°C.

(6) After renewed washing [as in (2) and (4)], 100 µl of substrate solution [9.9 ml 0.1 M sodium acetate buffer (pH 6.0), 100 µl tetramethylbenzidine (TMB)-substrate, 2 µl 37% H_2O_2] were added to each well. The peroxidase in the well converted the substrate to produce a blue colour. After 10-20 min the reaction was stopped by the addition of 1M H_2SO_4 (50 µl per well), and the colour was measured

spectrophotometrically at 450 nm. Calculation of the LPV-VP1 standard curve and conversion of the OD values of the samples into ng LPV-VP1 were performed with the aid of a program in the Photometer (Titertek Multiscan Plus MKII from Flow Laboratories, Meckenheim).

10.3. LPV binding test to BJA-B cells

Washed cells (1×10^6) were incubated with purified LPV particles (10 ng LPV-VP1) for 30 min at 37°C in 500 ml PBS (2×10^6 cells/ml). After centrifugation of the cells at 12,500 rpm for 2 min, the unbound virus in the supernatant was quantified with the LPV-VP1-ELISA. LPV binding was determined as the percentage of cell-associated virus relative to the total quantity of added virus (= 100%).

RESULTS

A. Inhibition of LPV-infectivity by pretreatmnent of BJA-B cells with *N*-pr panoyl- or *N*-butanoyl-D-mannosamine.

At a final analogue concentration of 10 mM, and pretreatment of BJA-B cells for 48 h, both the percentage of LPV-infected cells as determined in the direct immunofluorescence test, and LPV permissiveness, were decreased by between 89 and 95%. For the same period of treatment, 0.4 mM *N*-propanoyl-D-mannosamine caused a 50% decrease in LPV permissiveness. In cells preincubated for 48 h with 5 mM *N*-butanoyl-D-mannosamine, LPV-binding was decreased by 80%.

B. Increase of BKV-infectivity of Vero cells by pretreatment with *N*-propanoyl-D-mannosamine.

At a final concentration of 10 mM *N*-propanoyl-D-mannosamine, and pretreatment of Vero cells for 48 h, the number of BKV-infected cells increased seven-fold. If, after *N*-propanoyl-D-mannosamine treatment, the cells were then incubated with neuraminidase from *Vibrio cholerae* (200 mU/ml, 2h, 37°C), this infectivity was decreased by 80%.

A further important area of application of this discovery is the ability of analogues of neuraminic acid precursors (amino-sugars), with the general formula (II), to influence of the replication of human immunodeficiency virus type 1 (HIV-1) in MT-4 cells.

1. Materials and methods

The human T-cell line, MT-4, which is highly susceptible to infection by HIV-1 [Harada et al. (1985) *Science 229*, 563-566] was cultured in RPMI 1640 with addition

of 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, under grade L3 laboratory safety conditions.

HIV-1 (strain HTLV-III_B) inoculation virus (infectivity 1 x 10^6 IU/ml) was prepared from the supernatant of infected MY-4 cells as described by Popovic et al. (1984, *Science 224*, 497-501]. This HIV inoculation virus was aliquoted and stored at -70°C. HIV strain HTLV-IIIB is obtainable from the Division of AIDS, National Institute of Allergy and Infectious Diseases, 6003 Executive Boulevard, Bethesda, MD 20892 (catalogue number 398).

MT-4 cells (5 x 10^5) were cultured in 2 ml of medium in 12-well plates for 40 h in the presence of *N*-acyl-D-mannosamines. The neuraminic acid precursor analogues were dissolved in PBS, and their starting concentrations in the cultures were 2.5 and 5 mM. At the end of the incubation, the cells were washed in PBS, and counted. 1 x 10^6 living cells were placed in 2 ml medium and incubated with 100 ml HTLV-III_B inoculation virus for 3 h at 37°C. After washing once in 2 ml PBS, the cells were resuspended in medium and cultured for a further 24 h. The concentration of HIV-1 antigen was determined in the supernatants of these cultures with the aid of an ELISA (Vironostika HIV-Antigen ELISA Mikrosystems, Organon, Eppelheim) according to the instructions of the manufacturer.

2. Results

After treatment of MT-4 cells for 40 h with *N*-butanoyl-D-mannosamine at a concentration of 5 mM, the concentration of HIV antigen in the supernatant was decreased by 97%. When the concentration of the neuraminic acid precursor analogue was 2.5 mM, the concentration of HIVB antigen in the supernatant was decreased by 85%, compared with that from untreated cells.

Treatment of MT-4 cells with *N*-acetyl-D-mannosamine (40 h, 5 mM) served as a control. In this case, the concentration of HIV antigen in the supernatant was increased by 21%, compared with that from untreated cells.

Methods used

In vitro stimulation of lymphocytes

Peripheral blood lymphocytes (PBL) were obtained from the heparinized blood of tumour patients or from the "buffy coat" of healthy probands, using density gradient centrifugation according to Böyum.
Ficoll-Paque (15 ml; density 1.077 g/ml) in a centrifuge tube (50 ml) was carefully overlain with 30 ml blood, which had been previously diluted 1:2 to 1:3 with physiological saline. Centrifugation was performed for 30 min at 600 g. During centrifugation, the erythrocytes and granulocytes migrate through the Ficoll layer, whereas the mononuclear leukocytes become concentrated immediately above the separation medium. This cell layer was removed, suspended in physiological saline, and washed five times (200 g, 5 min). The mononuclear leukocytes were finally incubated for 16 h in plastic culture dishes in an incubation cabinet, in RPMI-1640 medium containing 10% FCS, 10^{-5} mol/l 2-mercaptoethanol and 2 mmol/l L-glutamine, in order to remove adherent monocytes. The suspension of non-adherent, peripheral blood lymphocytes was diluted to a cell density of 5×10^6/ml.
Cell vitality was determined by trypan blue staining: trypan blue solution (0.1%, 80 µl) was added to a cell suspension (20 µl); after mixing, vital cells were counted in a Neubauer counting chamber.
For the stimulation of lymphocytes, concanavalin A (4 µl/ml) was added to the cell suspension; maximal activity was observed after 72 h. For brief activation, concanavalin A (4 µg/ml) was added to the cell suspension, and cells were harvested after 1 h and washed twice (200 g, 5 min).

Cell lines used:
ATCC CCL 86 (Raji)
ATCC CCL 213 (Daudi)
ATCC CCL 229 (LoVo)
ATCC CCL 240 (HL-60)
ATCC CCL 243 (K-562)
ATCC CRL 1582 (Molt-4)
ATCC CRL 1593 (U-937)
ATCC HTB 22 (MCF-7)
ATCC HTB 38 (HT-29)

Uptake and incorporation of sugar analogues

The different sugar analogues were added to the cell suspensions in microtitre plates, to give a final concentration of sugar analogue between 0.05 mmol/l and 50 mmol/l. The cells were then incubated for 1 h to 72 h.

Determination of cell proliferation

Cell proliferation was determined with the ^{3}H-thymidine test. This test is based on the determination of the rate of incorporation of ^{3}H-thymidine into DNA, which is proportional to the viable cell count.

Cells were cultured in microtest plates (200 µl of cell suspension in each well). Sixteen hours before the end of the incubation, 0.037 MBq ^{3}H-thymidine was added to each well. Finally, 100 µl of each cell suspension were placed on filter paper disks, and the disks were allowed to dry in the air. Each air-dried disk was washed twice (30 min) with 10% trichloroacetic acid, twice (30 min) with 5% trichloroacetic acid, and finally twice (30 min) with 50% ethanol, then allowed to dry in the air. Radioactivity due to the incorporation of ^{3}H-thymidine was measured in a liquid scintillation counter.

Assay of adhesion to an extracellular matrix

The adhesion assay was carried out in 96-well microtitre plates, which were coated with different components of the extracellular matrix (fibronectin, vitronectin, collagen, laminin). The effect of sugar analogues on the ability of cells to adhere to matrix components was investigated. The relative number of bound cells was determined with the MTT test, the MUH test, and the crystal violet staining method.

The MUH test is based on the hydrolysis of the fluorogenic substrate, MUH (4-methylumbelliferyl-heptanoate), by esterases (Dotsika et al., 1987).

The MTT test is based on the conversion of tetrazolium salt into coloured formazan by the dehydrogenases of active mitochondria (Alley et al., 1988).

Assay of adhesion to endothelial cells

Microtitre plates coated with endothelial cells were incubated with ^{51}Cr-labelled tumour cells. The relative number of bound tumour cells was determined from the radioactivity of the lysate.

Detection of the presence of modified adhesion molecules

Adhesion receptors and the extent of their modification by sugar analogues were determined by flow-through cytometry in an FACScan, using monoclonal antibodies.

Assay of invasive potential

The effect of synthetic sugar analogues on the migratory behaviour of tumour cells was investigated in Boyden chambers, whose upper chambers were coated with components of the extracellular matrix (Repesch, 1989).

Determination of natural killer (NK) cell activity

The NK cell activity of peripheral mononuclear blood leukocytes was determined in a cytotoxicity test (performed on microtest plates) with ^{51}Cr-labelled target cells (K-562 cells). Release of ^{51}Cr was determined for different effector-target cell ratios (50:1, 25:1, 12.5:1, 6.25:1).

"Maximal release" of ^{51}Cr was determined by adding medium containing 2% Triton X-100 to the target cells instead of to the effector cells. "Spontaneous release" corresponded to the quantity of ^{51}Cr released by the target cells, when merely culture medium was added in place of effector cells.

Lytic units were calculated with aid of a program for the calculation of nonlinear regression analysis (Pross et al., 1948). The regression curve describes the dependency of specific ^{51}Cr release on the effector-target cell ratio, and therefore on the quantity of effector cells. One lytic unit (LU) corresponds to the number of effector cells necessary for the specific release of 30% of the ^{51}Cr activity.

Phagocytosis test

Monocytes were treated with sugar analogues. Both anti-D-sensitized erythrocytes and neuraminidase-treated sheep erythrocytes served as phagocytosis particles. The ability of granulocytes to take up latex particles within one hour was also tested.

In vivo experiments

Using experimental hepatomas, in particular Morris hepatoma 7777, investigations were performed to determine whether tumour growth is inhibited or prevented when tumour cells are injected into rats previously treated with sugar analogues. Alternatively, hepatoma cells treated with sugar analogues were injected into rats, and subsequent tumour growth studied.

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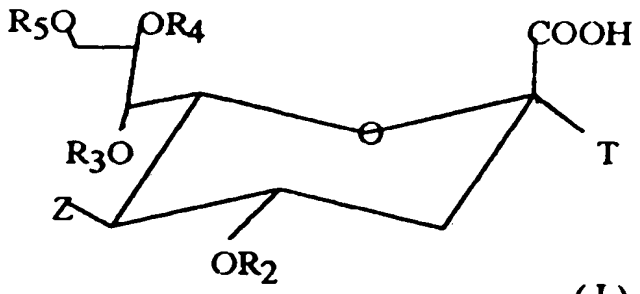
Patent claims

1. Glycoproteins with the general formula (I)



(I)

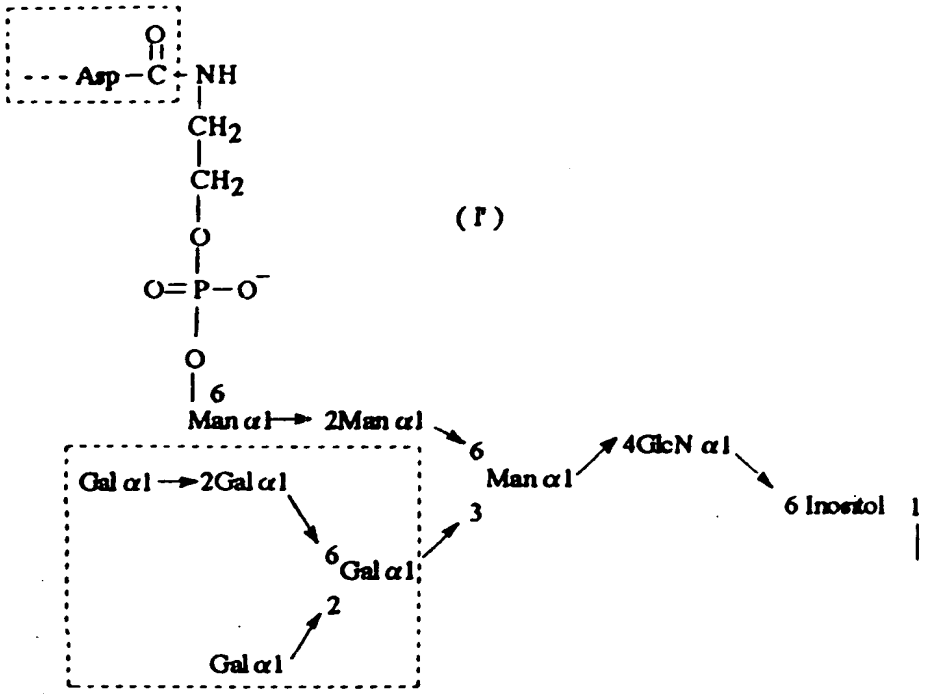
where:

Z represents $-R_1$, $-NH_2$, $-NHR_1$, $-NR_1R_{1'}$, $-N^+R_1R_{1'}R_{1''}$, $-OR_1$, $-SR_1$ or $-CR_1R_{1'}R_{1''}$;

R_1, $R_{1'}$, $R_{1''}$, R_2, R_3, R_4 and R_5 can be the same or different; each of these groups can be a hydrogen atom; a linear or branched alkyl residue with 1 to 20 carbon atoms (C_nH_{2n+2}, where n = 1 to 20), usually 1 to 7 carbon atoms; a linear or branched alkenyl residue with 3 to 20 carbon atoms (C_nH_{2n}, where n = 3 to 20; double bond on C2 to 19), usually 3 to 10 carbon atoms; a linear or branched alkynyl residue with 3 to 20 carbon atoms (C_nH_{2n-2}, where n = 3 to 20; triple bond on C2 to C19), usually 3 to 10 carbon atoms; an alkenyl or alkynyl residue with 2 or more double or triple bonds, with 4 to 10, usually 7 to 12 carbon atoms; an aryl residue with 6 to 20 carbon atoms, usually a phenyl residue; a linear or branched, saturated or mono- or multi-unsaturated acyl residue ($-CO-R_1$), with a total of 1 to 20, usually 1 to 7 carbon atoms, including its mono- or multi-hydroxylated analogues; an aroyl residue with 6 to 20, usually 6 to 10 carbon atoms; a carbonylamide residue of the formula $-CONH_2$, $-CONHR_1$, $-CONHk_1R_1$, or $-CONR_1R_{1'}R_{1''}$; a linear or branched, saturated or mono- or multi-unsaturated thioacyl residue ($-CS-R_1$), with a total of 1 to 20, usually 1 to 7 carbon atoms; or a thiocarbamide residue of the formula $-CS-NH_2$, $-CS-NHR_1$, $-CS-NR_1R_{1'}$, or $-CS-NR_1R_{1'}R_{1''}$; whereby each of the aforementioned residues, with the exception of H, can be singly or multiply substituted by halogens (fluorine, chlorine, bromine or iodine) and by hydroxy-, epoxy-, amino-, mercapto-, phenyl-, phenol or benzyl groups;

T is a mono-, di- or oligosaccharide residue (containing 5 to 230 carbon atoms) with up to 40 glycosidically linked, unbranched or branched sugar residues (with furanose and/or pyranose rings), linked *N*- or *O*-glycosidically to the polypeptide;

or is represented by glycoproteins of the general formula (I'), which serve as a glycophosphatidyl anchor:



(I')

where

Gal = galactose,

Man = mannose,

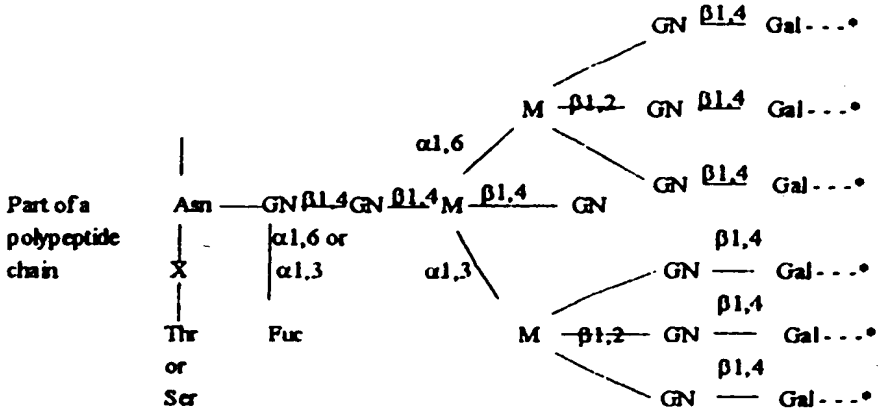
Asp = asparagine,

GlcN = glucosamine,

and in which GlcN can be replaced by an analogue of a neuraminic acid precursor (formula II).

2. Compounds of claim 1, characterised by the fact that Γ in formula (I) is represented either by:

a saccharide residue with *N*-glycan structure of formula (1a)



(Ia)

where

Gal = galactose,

GN = N-acetyl-D-glucosamine,

M = D-mannose,

Fuc = fucose,

Asn = asparagine,

X = any amino acid except proline,

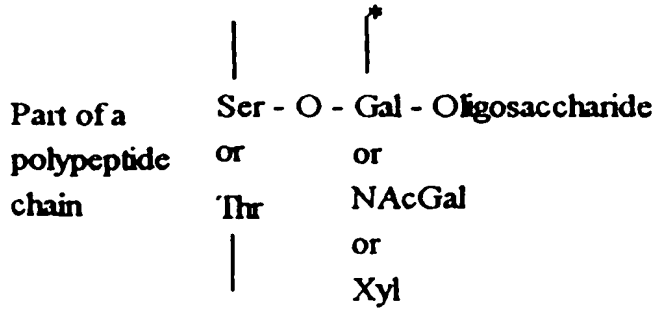
Thr = threonine,

Ser = serine,

* = attachment site of Γ (1 to 6 molecular residues),

in which both peripheral M residues can be sustituted by 1 to 3 trisaccharides;

or by an *O*-glycan structure of the general formula (Ib):

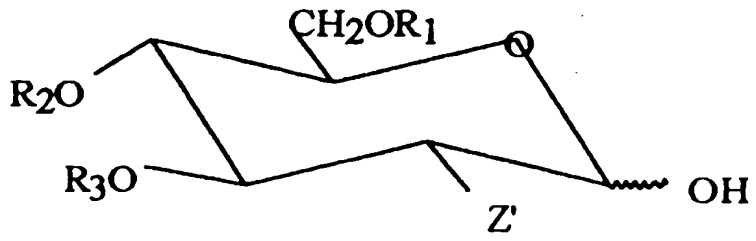


(Ib)

where
Gal = galactose
Thr = threonine
Ser = serine
Xyl = xylose
NAcGal = N-acetylgalactosamine,
* = attachment site of T,

and in which in the above formulae (Ia) and (Ib), galactose (Gal) can be replaced by 2-deoxy-galactose or 2-deoxy-2-halogenide (F, Cl, Br, I)-galactose.

3. Compounds of claims 1 and/or 2, characterised by the fact that when T is an *N*-glycan or an *O*-glycan, GN is a residue of the general formula (II):



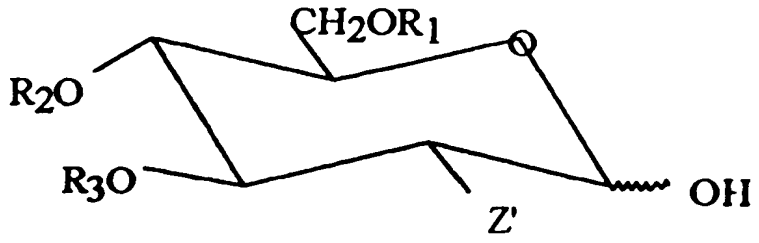
(II)

where R_1, R_2 and R_3 have the same meaning as in claim 1; Z' is the same as Z and can occupy an equatorial as well as an axial position; if Z' occupies the equatorial position, the axial position can be occupied by $-OR_1$.

4. Compounds of at least one of the claims 1 to 3, characterised by the fact that Z or Z' stands for NHR_1, where R_1 represents a propanoyl, butanoyl, pentanoyl, hexanoyl, heptanoyl or crotonoyl residue, or a mono- or multi-hydroxylated analogue of one of these residues.

5. Compounds of at least one of the claims 1 to 4, characterised by the fact that groups R_2 to R_5 are represented by H or CH_3.

6. Amino-sugars (analogues of neuraminic acid precursors) of the general formula (II):



(II)

in which Z', R_1, R_2 and R_3 have the same meaning as in claims 1 to 5.

7. Methods for the in vivo preparations of compounds in claims 1 to 6 by parenteral or enteral administration, to humans or animals, of a 2-deoxy-2-amino-mannose, -glucose or -galactose, in which the amino group contains the substituent R_1, and where the administered compound is usually *N*-propanoyl-, *N*-butanoyl-, *N*-pentanoyl-, *N*-hexanoyl-, *N*-heptanoyl- or *N*-crotonoyl-D-mannosamine.

8. Methods of claim 7, whereby the in vivo-synthesized glycoproteins of claims 1 to 5 are obtained (separated) by known methods and used therapeutically.

9. A pharmaceutical preparation for stimulation of the immune system, in particular T-lymphocytes, for protection against infection, for the treatment of a weak immune response, for the treatment tumours, including the process of metastasis, for treatment of infectious diseases (caused by viruses, bacteria, parasites, protozoa) and circulatory problems, in particular blood vessel occlusion and septicaemia, in humans and animals. This pharmaceutical preparation contains as active constituent at least one compound covered by claims 1 to 6, 7 (a product of the claimed method) and 8 (a product of the claimed method), if necessary in combination with other active constituents, as well as usual pharmaceutical vehicles and/or auxillary substances.

10. A pharmaceutical preparation for increasing the cytotoxic activity of natural killer cells (NK cells), for inducing an anti-tumour immune reaction in humans and animals. This parmaceutical preparation contains as active constituent at least one compound covered by claims 1 to 8, if necessary in combination with other active components, as well usual pharmaceutical vehicles and/or auxillary subsatnces.

11. A pharmaceutical preparation for increasing the phagocytic activity of granulocytes and monocytes, for the induction of an anti-tumour immune reaction in humans and animals. This pharmaceutical preparation contains as active constituent at least one compound covered by claims 1 to 8, if necessary in combination with other active components, as well as usual pharmaceutical vehicles and/or auxillary substances.

12. A pharmaceutical preparation for the in vivo modulation of neuraminic acid-dependent processes, which contains as active constituent at least one compound covered by claims 1 to 8, if necessary in combination with other active components, as well as usual pharmaceutical vehicles and/or auxillary substances.

13. A pharmacetical preparation for the inhibition of ligand binding to sialylated cell surface receptors (endothelial cells, thrombocytes, leukocytes), which contains as active constituent at least one compound covered by claims 1 to 8, if necessary in combination with other active components, as well as usual pharmaceutical vehicles and/or auxillary substances.

14. A pharmaceutical preparation for the inhibition of the binding of a pathogenic microorganism (virus, bacterium, parasite, protozoan) or a toxin to the sialylated receptor of a host cell, by in vivo modulation of neuraminic acids. This pharmaceutical preparation contains as active constituent at least one compound covered by claims 1 to 8, if necessary in combination with other active components, as well as usual pharmaceutical vehicles and/or auxillary substances.

15. A pharmaceutical preparation covered by claims 9 to 14, characterized by the fact that it contains at least one compound covered by claims 1 to 8 in a concentration of 0.01 to 50% by weight, usually in a concentration of 0.1 to 20% by weight, and in particular 2 to 10% by weight.

16. The use of compounds covered by claims 1 to 8 to stimulate the growth and differentiation of human and animal cells of the immune system, and to prevent adhesion of leukocytes, thrombocytes and tumour cells to blood vessel epithelial cells.

17. The use of compounds covered by claims 1 to 8 to stimulate the immune system, in particular T-lymphocytes, to protect against infection, for the treatment of a weak immune response, for the treatment of tumours, including the process of metastasis, for the treatment of infectious diseases (caused by viruses, bacteria, parasites, protozoa)

and circulatory problems, in particular blood vessel occlusion and septicaemia, in humans and animals.

18. The use of compounds covered by claims 1 to 8 for the inhibition of ligand binding to sialylated cell surface receptors.

19. The use of compounds covered by claims 1 to 8 to inhibit the binding of a pathogenic microorganism (virus, bacterium, parasite, protozoan) or toxin to the sialylated receptor of a host cell, by in vivo modulation of neuraminic acids.

20. The use of compounds covered by claims 1 to 8 for the biosynthetic preparation of ligands or receptors containing modified neuraminic acid, and the use of these modified ligands or receptors as physiological or pathological competitors of ligand-receptor interactions.

21. The use of glycoproteins covered by claims 1 to 4 for the in vitro modulation of the course of infection by human immune deficiency viruses (e.g. HIV-1 and HIV-2).

22. The use of compounds covered by claims 1 to 8 for the in vivo prevention of infection by human immune deficiency viruses (e.g. HIV-1 and HIV-2).

23. The use of compounds covered by claims 1 to 8 for the treatment of parasitic diseases, in particular trypanosomiasis, leishmaniasis, trichomoniasis, giardiasis, amoebiasis, malaria, pneumocytosis, schistosomiasis (Bilharzia) and echinococcosis